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**Title:** Local activation of uterine Toll-Like Receptor 2 and 2/6 decreases embryo implantation and affects uterine receptivity

**Running title:** Tlr2 and 2/6 activation affects uterine receptivity

**Summary sentence:** The activation of uterine innate immune system through TLR 2 and 2/6 affects the endometrial receptivity and embryo implantation in vivo in mice and in vitro in a human trophoblast-epithelial cells adhesion model.

**Key words:** Endometrium, Female Infertility, Implantation, Toll-Like Receptors (TLR), Trophoblast Spheroids.

**Authors:**

Javier Arturo Sanchez-Lopez<sup>1</sup>, Ignacio Caballero<sup>1</sup>, Mehrnaz Montazeri<sup>1</sup>, Nasim Maslehat<sup>1</sup>, Sarah Elliott<sup>1</sup>, Raul Fernandez-Gonzalez<sup>2</sup>, Alexandra Calle<sup>2</sup>, Alfonso Gutierrez-Adan<sup>2</sup> and Alireza Fazeli<sup>1,3</sup>

**Address:**

1. Academic Unit of Reproductive and Developmental Medicine, The University of Sheffield, Sheffield, UK.
2. Departamento de Reproduccion Animal, Instituto Nacional de Investigacion y Tecnología Agraria y Alimentaria (INIA), Madrid, Spain

3. All correspondence would be addressed to: [A.Fazeli@sheffield.ac.uk](mailto:A.Fazeli@sheffield.ac.uk), Direct Line: +44114-226 8195, Secretary: +44114-226 1075, Fax: +44114-226 8538

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**Abstract:**

Embryo implantation is a complex interaction between maternal endometrium and embryonic structures. Failure in implantation is highly recurrent and impossible to diagnose. Inflammation and infections in the female reproductive tract are common causes of infertility, embryo loss and preterm-labor. The current work describes how the activation of endometrial Toll-like receptor (TLR) 2 and 2/6 reduces embryo implantation chances. We developed a morphometric index to evaluate the effects of the TLR 2/6 activation along the uterine horn (UH). TLR 2/6 ligation reduced the endometrial myometrial and glandular indexes and increased the luminal index. Furthermore, TLR 2/6 activation increased the pro-inflammatory cytokines such as Interleukin (IL)-1 $\beta$  and Monocyte Chemoattractant Protein (MCP)-1 in UH lavages in the pre-implantation day and IL-1 Receptor Antagonist in the implantation day. The engagement of TLR 2/6 with its ligand in the uterine horn during embryo transfer severely affected the rate of embryonic implantation ( $45.00 \pm 6.49$  vs  $16.69 \pm 5.01\%$ ,  $p < 0.05$ , control vs test; respectively). Furthermore, this interference with the embryo

implantation process was verified using an in vitro model of human embryo implantation where trophoblast spheroids failed to adhere to a monolayer of TLR 2 and TLR 2/6 activated endometrial cells. The inhibition of TLR receptors 2 and 6 in the presence of their specific ligands, restored the ability of the spheroids to bind to the endometrial cells. In conclusion, the activation of the innate immune system in the uterus at the time of implantation interfered with the endometrial receptivity and reduced the chances of implantation success.

## 1 **Introduction**

2 Embryonic implantation is a critical event leading to a successful pregnancy[1]. The  
3 implantation process requires a complex orchestration of cellular and molecular events  
4 that include: expression of adhesion molecules, remodeling of the uterine extracellular  
5 matrix and an intricate crosstalk of hormones, cytokines and growth factors between the  
6 embryo and the endometrium[2-6]. Immune system plays an important role in the  
7 modulation of the mechanisms involved in implantation. Pregnancy represents an  
8 immunological contradiction in which a semi-allogeneic foreign entity, the embryo, is not  
9 rejected by the maternal immune system but accepted and nourished. This is achieved by  
10 several mechanisms, including the modulation of the maternal immune system by the pre-  
11 implantation embryo[7]. Consequently, any interference that may imbalance the immune  
12 system responses could result in embryo loss and infertility. In fact, many infertility  
13 problems in women are associated with infections of the upper female reproductive tract  
14 (FRT) compartments: endocervix, fallopian tubes and uterus. The pathogens responsible  
15 for sexually transmitted diseases (STD) such as *Mycoplasma genitalium*, *Neisseria*  
16 *gonorrhoeae* and *Chlamydia trachomatis* have been associated with infertility in  
17 women[1,8].

18 The FRT is able to respond against these pathogenic entities and initiate an immune  
19 response. The first recognition of pathogens in the FRT is mediated by the innate immune  
20 system of epithelial, resident dendritic and natural killer (NK) cells[3,7,9]. This innate  
21 response will later prime and instruct the adaptive immune system to initiate cellular and  
22 humoral responses against pathogens[7,9,10]. In order to detect the potential pathogens,  
23 endometrial epithelial cells express pattern recognition receptors (PRRs) to common

24 pathogen-associated molecular patterns (PAMPs). One of the main families of PRRs is  
25 TLR family[9,11]. They can recognize a great variety of PAMPs from bacterial, fungal,  
26 parasitical and viral origin. The TLR family is formed by 10 members where each  
27 respond to a specific ligand that promotes the secretion of a different subset of cytokines  
28 and chemokines[12-15]. TLR 2 and its heterodimers with TLR 1 and TLR 6 can sense a  
29 great variety of PAMPs from pathogens that might be present in the FRT and can be  
30 activated by peptidoglycans (PGN) from bacteria (*C. trachomatis*, *Staphylococcus*  
31 *aureus*, *N. gonorrhoeae*, *M. genitalium* lipoproteins)[16], yeast (*Candida albicans*  
32 phospholipomannan)[17] and parasites (*Trichomonas vaginalis* lipophosphoglycan)[18].  
33 The heterodimer of TLR 2 and TLR 6 has been found to recognize the macrophage  
34 activated lipoprotein derived from *Mycoplasma fermentans* (MALP-2)[19]. FSL-1 is a  
35 specific and potent ligand for the heterodimer TLR 2/6 and was synthesized based on  
36 MALP-2 structure by changing its amino acid sequence[20]. Upon this recognition, they  
37 initiate signaling pathways that end up in the activation of the transcription factors  
38 nuclear factor (NF)- $\kappa$ B or AP-1 that promote the activation of pro-inflammatory  
39 genes[21].

40

41 The expression of TLRs 1-10 has been observed in mammalian uterus and in different  
42 human endometrial epithelial cell lines[13]. This expression follows the menstrual  
43 hormonal cycle. The highest level of TLR expression in the human endometrium has  
44 been observed during the late secretory phase, where implantation of the embryo takes  
45 place[22]. Although TLRs have been observed to be involved in a number of pregnancy  
46 disorders such as preterm labor, early pregnancy loss and pre-eclampsia[23], not much is

47 known about their function during embryo implantation. Recent results from our  
48 laboratory have shown that activation of TLR5 by its ligand flagellin can interfere with  
49 trophoblast spheroids adhesion to epithelial cells in vitro[24].

50 The study of the human embryo implantation process in vivo is limited due to the  
51 anatomical and the physiological restrictions and the ethical and the legal issues  
52 surrounding experimentation with human embryos. For these reasons, research on the  
53 embryo implantation has opted to employ different animal model species. The human  
54 embryo implantation is an invasive process, which makes it different from other  
55 mammalian species. The closest in vivo model to human is the primate embryo  
56 implantation[25] but the manipulation of these species is also limited due to ethical  
57 responsibility. The murine model is a good animal model that shares similar implantation  
58 characteristics with human embryo implantation such as its invasiveness[26]. It also  
59 offers many advantages like the possibility to manipulate their gametes and estrous cycle  
60 (between 4 to 5 days) and a short gestation (Approx. 3 weeks)[27]. Implantation of the  
61 murine embryo will take place on metestrus (day 4.5) the equivalent of the late secretory  
62 phase in human. Another alternative for the study of embryo implantation are the in vitro  
63 models where different cell lines, primary cells or tissue explants mimic both  
64 endometrium and embryo[28]. The selection of the appropriate cell lines is a fundamental  
65 issue to be considered due to the differences in endometrial and trophoblast cell  
66 developmental stages[28]. In the current investigation, we employed an in vitro model of  
67 human implantation, where the endometrium was simulated using the RL95-2 cell line  
68 which has been reported to maintain the epithelial polarization, express adhesion  
69 molecules and microfilaments in the apical surface and being responsive to hormones and

70 secretion of cytokines[29]. The breast carcinoma epithelial cells MCF7 were also  
71 employed in our investigations to observe if the trophoblasts were able to interact with  
72 another epithelial cell line. MCF7 maintain epithelial cell characteristics and are  
73 responsive to hormonal stimulation, hence they were used as another hormone responsive  
74 reproductive tissue from a non-endometrial origin[30]. The human embryo was simulated  
75 with multi-cellular spheroids from choriocarcinoma trophoblast cell line JAr. This cell  
76 line has been employed in different studies for its ability to establish adhesive interactions  
77 with endometrial cell lines in short periods of co-incubation[31].

78

79 In the current investigation, we determined if the activation of the TLR 2 could influence  
80 the implantation of the embryo. The problem was explored using two different  
81 approaches. Initially we used an in vivo murine embryo implantation model, to assess if  
82 the activation of TLR 2/6 in the UH may affect uterine morphology and secretory profile  
83 during implantation period as well as embryonic implantation. Thereafter, we  
84 corroborated these observations in an in vitro human implantation model. In summary,  
85 the activation of TLR 2/6 in vivo decreased the murine embryo implantation rate with  
86 significant changes in tissue morphology and protein secretion. In addition, the in vitro  
87 adhesion of trophoblast spheroids to the endometrial cells was affected specifically by the  
88 activation of the endometrial TLRs.

89

## 90 **Materials and Methods**

### 91 **Embryo transfer experiments**

#### 92 *Mice superovulation and mating*

93 C57/CBA mice were obtained from Harlan Laboratories. All mice used in the  
94 experiments were housed under controlled temperature conditions of 22°C with a 14/10 h  
95 light/dark cycle, 40-60% relative humidity and free access to water and food. Females  
96 were kept in cages of 1000 cm<sup>2</sup> in groups of 8-10. Males were kept individually in 250  
97 cm<sup>2</sup> cages. All animal experiments were performed in accordance with Institutional  
98 Animal Care and Use Committee guidelines from the INIA (Instituto Nacional de  
99 Investigación y Tecnología Agraria y Alimentaria) and in adherence with guidelines  
100 established in the Guide for Care and Use of Laboratory Animals as adopted and  
101 promulgated by the Society for the Study of Reproduction. Females were injected i.p.  
102 with 7.5 IU of equine chorionic gonadotropin (eCG; Foligon 500, Intervet), followed 48 h  
103 later by 7.5 IU of human chorionic gonadotropin (hCG; Veterin Corion, Equinvest).  
104 Thereafter each female was placed in a male cage (2:1) for mating. On the next day (day  
105 0.5 8:00-10:00 h) the females with vaginal plug were separated from the males. The  
106 selected mice were euthanized by cervical dislocation 3.5 days post-mating (embryos  
107 reached blastocyst stage). Both uterine horns were dissected and placed in a petri dish in  
108 pre-warmed M2 media. The lumen of each horn was rinsed with M2 media (M7167,  
109 Sigma, Dorset, UK), using a 1ml syringe with a 30G needle. The blastocysts were  
110 collected under a stereoscopic microscope using a glass micropipette and rinsed twice in  
111 M2 media. Finally they were rinsed once in pre-warmed K<sup>+</sup> modified simplex optimized  
112 medium (KSOM, KSOMaa Evolve, ZEKS-050, Zenith Biotech) for at least 30 min until

113 embryo transfer. The blastocysts were rinsed in warmed up M2 media and placed in a  
114 transfer pipette.

115

### 116 ***Foster mice preparation***

117 The foster CD1 mice were obtained from Harlan Laboratories. The females bedding was  
118 replaced with male bedding to initiate the estrous cycle. At the start of the estrus phase of  
119 the cycle (two days later), the females were mated with vasectomized males to induce the  
120 pseudo-pregnancy state and prepare the reproductive tract to receive the embryos. The  
121 foster pseudo-pregnant mice were selected by the presence of a vaginal plug. On day 3.5  
122 post-mating, females were anesthetized by inhalation of Isoflurane and kept on a warm  
123 plate during the surgery. The left uterine horn was exposed and the top of the utero-tubal  
124 junction was carefully pierced with the tip of a 30G needle where a transfer pipette  
125 loaded with the blastocysts was inserted. The pipette reached the uterine horn (UH)  
126 through the opened utero-tubal junction and delivered the blastocysts. The foster mice  
127 were kept in separate cages with tags, identifying the date of embryo transfer, number of  
128 embryos transferred and treatment. Food and water were supplied ad libitum.

129

### 130 **Histological evaluation of the murine UHs**

#### 131 ***Sample collection and storage***

132 The foster mice were euthanized by cervical dislocation. UHs were dissected and placed  
133 in petri dishes. The UHs were stored in 10% paraformaldehyde and dehydrated in a series  
134 of ethanol dilutions (70, 90 and 100%) for fixation. Finally UHs were sectioned in two  
135 and embedded in paraffin. The paraffin blocks were cut in transversal sections of 4  $\mu\text{m}$

136 using a microtome and fixed to normal microscope slides. The sections on the slides were  
137 then stained using the conventional Haematoxylin & Eosin (H&E) method. Briefly, wax  
138 was removed by rinsing twice in xylene for 5 min. Tissue was hydrated in series of  
139 ethanol dilutions (Twice in 99, 95 and 70%) and tap water for 5 min. The slides were  
140 stained with Gill II Haematoxylin (VWR, Lutterworth, UK) for 1 min and rinsed with tap  
141 water for 3 min. The slides were stained in 1% aqueous Eosin (VWR) with 1% Calcium  
142 Carbonate (Sigma) for 5 min and rinsed in tap water for 30 sec. The sections were  
143 dehydrated in serial dilutions of ethanol (70 and 95% for 10 sec and twice 99% for 30  
144 sec) and twice in Xylene (1 and 3 min). Finally the coverslip was fixed with DPX  
145 mountant (VWR).

146 The cross sections in the slides were imaged using light microscopy (Olympus CKX41;  
147 Southend-on-sea, UK) and captured with the NIS elements software (Version F3.0 SP4;  
148 Nikon, Surrey, UK). The changes of the development of the uterine decidua due to estrus  
149 cycle were observed.

150

### 151 *Morphometric analysis of the UH*

152 The images of 10 cross-sections were captured using the 20x objective and measured  
153 using the software ImageJ (NIH, Bethesda, MD, USA). This step was taken to ensure the  
154 consistency of the measurements along the length of the horn. The scale for ImageJ area  
155 measurements was set using the scale bar (500  $\mu\text{m}$ ) given by the capture software. Each  
156 respective area was measured by outlining it with the free hand selection tool and the area  
157 obtained using the measure option. For each UH cross-section the total area ( $A_T$ ;  
158 myometrium, endometrial stroma and epithelium and lumen), endometrial area ( $A_{e1}$ ;

159 including lumen), luminal area ( $A_L$ ) was measured and the number of glands (nG) was  
 160 counted. The endometrial area ( $A_E$ ) and the myometrial area ( $A_M$ ) were calculated as  
 161 follows:

$$162 \quad A_E = A_{el} - A_L$$

$$163 \quad A_M = A_T - A_{el}$$

164 Then the myometrial index ( $M_i$ ), endometrial index ( $E_i$ ), luminal index ( $L_i$ ) and gland  
 165 index ( $G_i$ ) were calculated as follows:

$$166 \quad M_i = \frac{A_M}{A_T}$$

$$167 \quad E_i = \frac{A_E}{A_T}$$

$$168 \quad L_i = \frac{A_L}{A_T}$$

$$169 \quad G_i = nG \quad E_i$$

170

## 171 **Cytokine measurement**

### 172 *Sample collection and storage*

173 The foster mice were euthanized by cervical dislocation. UHs were dissected and placed  
 174 in petri dishes. The UHs were rinsed with 200  $\mu$ l of M2 media using a 1 ml syringe. The  
 175 media was collected in 0.5 ml centrifuge tubes and stored at  $-80^\circ\text{C}$ .

176

### 177 *Cytokine Arrays*

178 The flushed media samples were analyzed using the Mouse Cytokine Array Panel A kit  
179 (R&D Systems, Abingdon, UK) for the production of 40 soluble proteins: B-Cell Chemo-  
180 attractant (BCL), Complement component 5a (C5a), Granulocyte Colony Stimulating  
181 Factor (G-CSF), Granulocyte – Macrophage Colony Stimulating Factor (GM-CSF), T-  
182 Cell Activation-3 (TCA-3), Eotaxin, Soluble Intercellular Adhesion Molecule-1 (sICAM-  
183 1), Interferon (IFN)- $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-  
184 13, IL-12 p70, IL-16, IL-17, IL-23, IL-27, Inflammatory Protein (IP)-10, Interferon-  
185 inducible T-cell Alpha Chemo-attractant (I-TAC), Keratinocyte Chemo-attractant (KC),  
186 Macrophage Colony-stimulating Factor (M-CSF), MCP-1, MCP-5, Monokine Induced by  
187 Gamma-interferon (MIG), Macrophage Inflammatory Protein (MIP)-1 $\alpha$ , MIP-1 $\beta$ , MIP-2,  
188 Regulated upon Activation Normal T-cell Expressed and presumably Secreted  
189 (RANTES), Stromal cell Derived Factor (SDF)-1, Thymus and Activation Regulated  
190 Chemokine (TARC), Tissue Inhibitor of Metalloproteinases (TIMP)-1, Tumor Necrosis  
191 Factor (TNF)- $\alpha$  and Triggering Receptor Expressed on Myeloid cells (TREM)-1. The  
192 samples were incubated overnight with the membranes and the detection antibodies at  
193 8°C on a rocking platform. The membranes were rinsed with wash buffer twice for 10  
194 min and incubated with 2 ml of Streptavidin-HRP for 30 min. The membranes were  
195 rinsed twice in wash buffer and 0.5 mL of chemiluminescent reagent was used to develop  
196 the array. Images of the membranes were captured in an image analyzer after 5 min  
197 exposure.

198

199 *Cytokines and chemokine quantification*

200 The chemokine MCP-1 and cytokine IL-1 $\beta$  were evaluated in the UH flushing  
201 samples by flow cytometry using a Cytokine Bead Array (CBA, BD Biosciences, Oxford,  
202 UK). Briefly, 50  $\mu$ l of the flushed media samples were incubated with 50  $\mu$ l of different  
203 cytokine detection beads mix, 50  $\mu$ l of the Phycoerythrin (PE) detection reagent and  
204 incubated for 3h. The samples were washed with 300  $\mu$ l of the provided wash buffer and  
205 centrifuged at 200 x g for 5 min. The pellets were re-suspended in 300  $\mu$ l of wash buffer  
206 and analyzed in a FACSArray cytometer (BD Biosciences). The cytokine IL-1RA was  
207 measured using an ELISA kit (PeproTech; London, UK). Briefly 100  $\mu$ l of the flushed  
208 media samples were incubated at room temperature in the anti-IL-1RA antibody pre-  
209 coated 96 well plate for 2h. The plate was rinsed 4 times with washing buffer and 100  $\mu$ l  
210 of the secondary biotinylated antibody (500 ng/ml) were added and co-incubated for 2 h.  
211 The plate was rinsed 4 times with the washing buffer and 100  $\mu$ l of the avidin peroxidase  
212 (1:200) were added and incubated for 30 min. Finally the plate was rinsed 4 times with  
213 washing buffer and 100  $\mu$ l of the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-  
214 sulphonic acid)) liquid substrate were added. The optical density was measured in a plate  
215 reader at 405 nm with wavelength correction set at 650 nm.

216

## 217 **In vitro adhesion assay of trophoblast spheroids to the endometrial cells**

218

### 219 *Epithelial cell lines culture*

220 RL95-2 cells were cultured in T75 flasks at 37°C in Dulbecco Modified Eagle Medium  
221 (DMEM)/F-12 (Sigma) supplemented with 1% Penicillin/Streptomycin (P/S; Sigma), 5  
222  $\mu$ g/ml Insulin (human recombinant insulin; Gibco Invitrogen, Denmark; cat. No. 12585-

223 014), 1% L-Glutamine (Sigma) and 10% Fetal Bovine Serum (FBS; Sigma) at 37°C in  
224 5% CO<sub>2</sub> atmosphere until reaching confluency.

225 MCF7 cells grown in DMEM/F-12, supplemented with 10% FBS, 1% P/S, 1% L-  
226 Glutamine and 160 ng/ml Insulin in a T75 flask at 37°C in 5% CO<sub>2</sub> atmosphere. At  
227 confluency, cells were washed with Dulbecco Phosphate-Buffered Saline (DPBS;  
228 Sigma), harvested using Trypsin-EDTA (Sigma) and pelleted by centrifugation at 300 x g  
229 for 4 min. Approximately 5x10<sup>5</sup> RL95-2 cells and 2.5x10<sup>5</sup> MCF7 cells were transferred  
230 to each well of a 12-well plate and incubated at 37°C for 3-4 days.

231

### 232 *Formation of JAr cells spheroids*

233 JAr cells were grown in RPMI 1640 (Sigma), supplemented with 10% FBS, 1% P/S and  
234 1% L-Glutamine in a T75 flask at 37°C in 5% CO<sub>2</sub> atmosphere. At confluency, cells were  
235 washed with DPBS (Sigma), harvested using Trypsin-EDTA (Sigma) and pelleted by  
236 centrifugation at 300 x g for 4 min. To mimic the blastocyst, multicellular spheroids were  
237 formed from the JAr cells. One million JAr cells were counted with a haemocytometer  
238 and transferred to 5 ml of supplemented RPMI media in 60 x 15 mm Petri dishes. The  
239 spheroids were formed when the cell suspension was spun overnight on a gyratory shaker  
240 (JKA, MTS 2/4 digital, Staufen, Germany) at 280 rpm in a humid atmosphere and 5%  
241 CO<sub>2</sub> at 37°C. Approximately 2000 spheroids were obtained per petri dish (approximately  
242 150-200 µm diameter).

243

### 244 *Adhesion of JAr spheroids to endometrial cells*

245 Fifty JAr spheroids were picked and gently delivered to each well of a 12-well plate with  
246 a confluent monolayer of the epithelial cells. They were co-cultured in supplemented  
247 DMEM-F12 at 37°C and 5% CO<sub>2</sub>. After co-incubation, the initial number of JAr  
248 spheroids was confirmed by visual examination under a stereoscopic microscope. To  
249 remove non-adherent JAr spheroids from the epithelial cell monolayers, the 12-well  
250 culture plate was washed using a horizontal shaking device (Labman Automation LTD,  
251 Stokesley, UK). The plate was incubated for 4 min at 200 rpm and the media was  
252 discarded. Each well was filled with 1 ml of PBS containing Ca<sup>2+</sup> and Mg<sup>2+</sup> (PBS/Ca-Mg;  
253 Gibco Invitrogen; UK) and then set to shake for 4 min at 200 rpm. Finally, the PBS/Ca-  
254 Mg was discarded. The final number of spheroids was counted under the microscope and  
255 the results expressed as the percentage of spheroids from the initial number of spheroids  
256 counted before washing.

257

258 Viability assessment of the epithelial cells

259 The RL95-2 and MCF7 cells were grown in 12-well plates until total confluence. The  
260 media was replaced with serum free media before they were either activated or not with  
261 PGN and FSL-1 (Invivogen, Toulouse, France). The cells were harvested with 50 µl of  
262 trypsin EDTA to detach them from the wells, collected in 500 µl of media and pelleted by  
263 centrifuging at 300 g for 5 min. The cells were then resuspended in 200 µl of PBS and  
264 divided in two 5 ml cytometry tubes. One sample was used as autofluorescence and the  
265 other used stained with propidium iodide (PI; Life technologies, Paisley, UK) 3 µM and  
266 captured immediately. The samples were read in a FACSCalibur cytometer (BD)  
267 capturing 1x10<sup>4</sup> events and the percentage of PI positive events (death cells) was

268 registered. The results are expressed as percentage of live cells and were compared using  
269 a one-way ANOVA and  $p < 0.05$  was considered significant.

270

## 271 **Experimental Design**

272

### 273 **Evaluation of the murine UH after TLR 2/6 activation**

#### 274 *Morphological and morphometrical assessment and evaluation of the UH protein* 275 *secretion after TLR 2/6 activation*

276

277 The foster Swiss mice were mated with vasectomized males to induce the pseudo-  
278 pregnancy state. On day 3.5 post-mating, the utero-tubal junction of the left UH was  
279 pierced with the tip of a 30G needle and a transfer pipette was inserted through the  
280 opening to deliver 10  $\mu$ l of FSL-1 (0.1  $\mu$ g/ $\mu$ l;  $n = 16$ ) or saline solution as a control group  
281 ( $n = 10$ ). Mice were sacrificed 4h later (pre-implantation day) or on day 4.5 (implantation  
282 day) and the uterine horns collected. The inflammatory reaction in the UH was assessed  
283 by morphological and morphometric evaluation of the H&E stained samples. The flushed  
284 media samples from the UHs were pooled in four different groups: Ctrl 4h, FSL-1 4h,  
285 Ctrl 24h and FSL-1 24h. The protein production was assessed using the semi-quantitative  
286 Mouse Cytokine Array Panel A kit (R&D Systems) for the presence of 40 different  
287 cytokines. The profile of protein production was compared between the experimental  
288 groups. Furthermore, the concentrations of the cytokines IL-1 $\beta$ , and MCP-1 were  
289 assessed in each individual sample by CBAs (BD) and IL-1RA with an ELISA kit  
290 (PeproTech) and data were log transformed to normalize distribution.

291

292 ***Evaluation of the effect of TLR 2/6 activation on embryo implantation***

293 The Swiss foster mice were prepared for embryo transfer as mentioned above. The utero-  
294 tubal junction of the left UH in each mice was pierced with a 30G needle to make an  
295 opening and using a transfer pipette infused with a drop of 10 µl of saline solution (n=15)  
296 or FSL-1 (0.1 µg/µl; n=16) followed by a gap of air and a drop containing the 12  
297 blastocysts. After 15 days the foster mice were euthanized by cervical dislocation. The  
298 uterine horns were dissected and the number of embryos implanted and fetuses resorbed  
299 was registered (Fig. 1).

300

301 **Effect of the activation of epithelial TLR 2 and TLR 2/6 on the adhesion of**  
302 **trophoblast spheroids.**303 ***Adhesion of trophoblast spheroids to epithelial cells treated with different***  
304 ***concentrations of TLR 2 and 2/6 ligands***

305 To determine whether TLR 2 and TLR 2/6 activation is affecting the trophoblast adhesion  
306 to the epithelial cells, we evaluated the effect of different concentrations of Peptidoglycan  
307 (PGN) and FSL-1 on the epithelial cells. The RL95-2 and MCF7 cells were grown in 12-  
308 well plates until total confluence. The media was replaced with serum free media before  
309 they were either activated or not with PGN and FSL-1 (Invivogen, Toulouse, France).  
310 The epithelial cells were then stimulated with different concentrations of PGN (0, 5, 10  
311 and 20 µg/ml) or FSL-1 (10, 100 and 200 ng/ml) for 24 h. JAr spheroids were delivered  
312 onto the confluent monolayers and co-incubated for 1h. Adhesion was assessed as  
313 described.

314

315 *Adhesion of trophoblast spheroids to stimulated epithelial cells at different time*  
316 *intervals*

317 To understand if co-incubation duration is affecting the outcome of trophoblast binding to  
318 the epithelial cells, the cells were pre-incubated with 10 µg/ml of PGN or 100 ng/ml of  
319 FSL-1 for 24h. There after 50 JAr spheroids were delivered onto the confluent epithelial  
320 cell monolayers in 12-well plates and co-incubated for 0.5, 1, 2 or 4 h. The adhesion was  
321 assessed as described before.

322

323 *Effect of the inhibition of epithelial TLR activation on trophoblast spheroid adhesion*

324 To understand the effect of TLR 2 inhibition in endometrial cells on trophoblast  
325 spheroids binding to endometrial cells, the epithelial cells were either pre-treated or not  
326 with a monoclonal antibody against TLR 2 (2 µg/ml, neutralizing IgA monoclonal,  
327 Invivogen) or anti-TLR 6 monoclonal antibody (mouse IgG1, 10 ng/ml, Invivogen) 1 h  
328 before the addition of PGN (5 µg/ml) or FSL-1 (10 ng/ml) respectively. After 24 h, 50  
329 JAr spheroids were delivered onto the monolayer and co-incubated for 30 min. Non-  
330 adherent spheroids were removed and the percentage of adhered spheroids was  
331 determined.

332

333 *The effect of TLR activation on the viability of the epithelial cells*

334 To determine whether TLR 2 and TLR 2/6 activation is affecting the viability of the  
335 epithelial cells; we treated the cells with different concentrations of PGN and FSL-1. The  
336 epithelial cells were then stimulated with different concentrations of PGN (0, 5, 10 and

337 20 µg/ml) or FSL-1 (10, 100 and 200 ng/ml) for 24 h. The viability of the cells was  
338 verified by PI staining in the FACS Calibur cytometer.

339

#### 340 *Statistical analysis*

341 The results were expressed as mean ± SEM. Statistical analysis was performed using  
342 Statistica (V7; Statsoft UK, Letchworth, UK). The comparison of two experimental  
343 groups was performed with the non-parametric Mann-Whitney U test. The concentration  
344 of the cytokines measured by CBA and ELISA were log transformed to normalize  
345 distribution. Multiple groups were compared with a one-way ANOVA and a Bonferroni  
346 multiple comparison post-test. The effects of FSL-1 and PGN treatments on the co-  
347 incubation kinetics were compared with a two-way ANOVA test and a Bonferroni post-  
348 test to compare effects between non-stimulated control and treated cells.  $P \leq 0.05$  was  
349 considered to be significant.

350

#### 351 **Results:**

352

##### 353 *Local FSL-1 administration in uterus induced morphological changes in UH.*

354 The saline treated UHs on the pre-implantation day (4h post-treatment) retained the  
355 morphology of a normal UH. The myometrium was dense and endometrial stroma filled  
356 with secretory glands. The endometrial lumen was pseudo-stratified and convoluted,  
357 showing a small luminal space. Finally, an abundant secretory activity in the endometrial  
358 luminal cells and glands could be observed (Fig 2A). In contrast to this, the FSL-1 treated  
359 UH presented an atrophic morphology. The thickness of the myometrium and stroma was

360 reduced as well as the number of glands observed. The FSL-1 treated epithelial  
361 endometrial cells morphology changed from columnar pseudo-stratified to simple  
362 columnar/cuboidal shape. Nevertheless, some secretory activity could still be observed  
363 (Fig 2A). The *Mi*, *Ei* and *Gi* were significantly higher in the saline group compared to the  
364 FSL-1 treated group (Fig. 2B). In contrast, the lumen was smaller and more convoluted in  
365 the saline than in the FSL-1 treated (Fig. 2B). In controls, the morphology of the UHs on  
366 the implantation day (24h post-treatment) was similar to the ones at estrus. In addition to  
367 this, the stroma was loose and filled with edema, a characteristic of a decidualized  
368 endometrium. The presence of vacuoles in the cytoplasm of luminal and glandular  
369 epithelial cells confirmed a high secretory activity at this stage. The 24 h FSL-1 treatment  
370 induced an atrophica of the UHs decidua (Fig. 2C). This could be observed in the  
371 reduction of the *Mi* and *Ei* of the FSL-1 treated UHs compared to the saline treated UHs  
372 (Fig. 2D).

373

374 ***FSL-1 treatment of the UHs increased the production of pro-inflammatory cytokines.***

375 Sixteen proteins were detected 4h after treatments and 17 proteins were detected 24h after  
376 treatments in the cytokine array. The FSL-1 treatment of the UH on the pre-implantation  
377 day, increased the production of TNF- $\alpha$ , TIMP-1, MCP-1, M-CSF, KC, IL-16, IL-1ra, IL-  
378 1 $\beta$  and IFN- $\gamma$  (Fig. 3A). From these proteins, the highest difference between FSL-1 and  
379 saline treatments was observed in MCP-1, IL-1ra and IL-1 $\beta$ . FSL-1 treatment for 24 hrs  
380 increased significantly the production of MCP-1, IL-17, IL-16, IL-13, IL-7, IL-4, IL-1ra,  
381 IL-1 $\beta$ , IL-1 $\alpha$  and C5a (Fig. 3B). Based on these analyses, each individual sample was  
382 analyzed again by CBA for the production of IL-1 $\beta$ , MCP-1 and by ELISA for IL-1RA.

383 The FSL-1 treatment of the tissue for 4h considerably increased the production of IL-1 $\beta$ ,  
384 MCP-1 however the change in IL-1RA production was minimal (Fig. 3C). The  
385 production of IL-1 $\beta$  and MCP-1 after 24h of FSL-1 treatment was lower and no  
386 difference was observed between the different treatments. In contrast, this treatment  
387 significantly increased IL-1RA production (Fig. 3D).

388

389 ***Activation of TLR 2/6 in the murine uterine horn affects embryo implantation.***

390 The activation of TLR 2/6 with FSL-1 severely reduced the number of total implantations  
391 in the UH compared to the saline treated horns ( $45.00 \pm 6.49$  vs  $16.69 \pm 5.01\%$ ,  $p < 0.05$ ;  
392 Fig. 4A). The number of total implantations included the embryos that developed to  
393 fetuses and those that were resorbed (implanted embryos that could not develop; Fig. 4B).  
394 The percentage of developed fetuses decreased after the administration of FSL-1 to the  
395 UH ( $45.56 \pm 6.79$  vs  $26.04 \pm 6.84\%$ ,  $p < 0.05$ ; Fig. 4C). On the other hand, we observed  
396 an increase in the percentage of resorbed fetuses in the FSL-1 treated group compared to  
397 the saline group ( $4.44 \pm 1.79$  vs  $14.17 \pm 3.45\%$ ,  $p < 0.05$ ; Fig. 4D).

398

399 ***The activation of epithelial TLR 2 and TLR 2/6 decreased the adhesion of trophoblasts***  
400 ***spheroids in vitro regardless of the co-incubation time.***

401 The trophoblast spheroids adhered to the cells as soon as 30 min after their introduction  
402 to the wells and this adhesion was always higher in the RL95-2 cells ( $63.4 \pm 4.38\%$  and  
403  $37.35 \pm 3.02\%$  to MCF7 cells). The effect of the treatment of the epithelial cells with 100  
404 ng/ml of FSL-1 for 24 h on the adhesion of the JAr spheroids was significant for RL95-2  
405 ( $p < 0.05$ ) and MCF7 cells ( $p < 0.0001$ ) as well as the effect of the co-incubation time ( $p$

406 < 0.0001 for RL95-2 and  $p < 0.0001$  for MCF7). The FSL-1 stimulation was consistent  
407 throughout the time as no interaction was observed in both cell lines ( $p = 0.6473$  for  
408 RL95-2 and  $p = 0.8718$  for MCF7 cells; Fig. 1A and B). The JAr spheroid adhesion was  
409 found significantly different between the non-stimulated control and FSL-1 stimulated  
410 MCF7 cells at 30 min ( $37.35 \pm 3.02$  vs  $26.56 \pm 2.59\%$ ,  $p < 0.01$ ) and 4 h ( $73.01 \pm 2.23$  vs  
411  $62.28 \pm 2.053\%$ ,  $p < 0.01$ ). The treatment of the cells with PGN was also consistent  
412 throughout the stimulation time as no interaction was observed ( $p = 0.5527$  for RL95-2  
413 and  $p = 0.5663$  for MCF7). We observed a significant effect of the treatment of the cells  
414 with  $10 \mu\text{g/ml}$  of PGN on the adhesion of JAr spheroids adhered in both RL95-2 ( $p <$   
415  $0.0001$ ) and MCF7 cells ( $p < 0.0001$ ). The effect of time on the adhesion of the spheroids  
416 was also found to be significant in both cell lines ( $p < 0.0001$  for both RL95-2 and  
417 MCF7; Fig. 5A and B). The adhesion of the JAr spheroids to the non-treated RL95-2  
418 cells differ significantly from the PGN treated cells at 1 h ( $73.79 \pm 2.85$  vs  $64.1 \pm 3.9$ ,  $p <$   
419  $0.05$ ) and 2h of co-incubation ( $89.05 \pm 2.02$  vs  $76.39 \pm 2.54$ ,  $p < 0.01$ ). The same effect  
420 was observed in the MCF7 cells at 1 h ( $73.01 \pm 2.43$  vs  $67.95 \pm 3.23$ ,  $p < 0.05$ ) and 2 h of  
421 co-incubation with the JAr spheroids ( $84.19 \pm 3.11$  vs  $71.34 \pm 4.26$ ,  $p < 0.01$ ).

422

423 ***Trophoblast spheroid decreased adhesion to the epithelial monolayer proportional to***  
424 ***the TLR ligand concentration.***

425 The 24h stimulation of RL95-2 and MCF7 cells with 100 and 200 ng/ml of FSL-1 ( $68.11$   
426  $\pm 4.83$  and  $61.07 \pm 4.52 \%$  for RL95 and  $41.66 \pm 2.82$  and  $40.21 \pm 3.36 \%$  for MCF7)  
427 significantly decreased the adhesion of the JAr spheroids to the monolayer compared to  
428 the non-stimulated control ( $83.46 \pm 2.685 \%$ ,  $p = 0.0012$  for RL95 and  $58.58 \pm 2.43 \%$ ,  $p$

429 < 0.0005 for MCF7) after one hour of co-incubation (Fig 6A and B). A similar effect was  
430 observed with PGN stimulation. The RL95-2 PGN stimulation with 20 µg/ml  
431 significantly decreased JAr spheroid adhesion compared to the non-stimulated control  
432 ( $57.09 \pm 3.83$  vs  $72.92 \pm 3.74$  %,  $p < 0.05$ ; Fig. 6A). In MCF7 5, 10 and 20 µg/ml of PGN  
433 significantly decreased JAr spheroid adhesion ( $57.05 \pm 3$ ,  $47.04 \pm 1.23$  and  $50.19 \pm 2.86$   
434 % respectively) compared to the non-stimulated control ( $68.25 \pm 2.71$  %,  $p < 0.0001$ ; Fig.  
435 6B).

436

437 ***The pretreatment of the endometrial cells with TLR blocking antibodies could restore***  
438 ***the spheroid adhesion.***

439 Furthermore, we confirmed the specificity of the TLR effect by blocking their activation  
440 with monoclonal antibodies. The pre-treatment with a monoclonal anti-TLR 6 antibody  
441 followed by FSL-1 stimulation, restored the percentage of adhered JAr spheroids to  
442 RL95-2 cells ( $59.71 \pm 1.91$ %) and MCF7 cells ( $30.70 \pm 4.54$ %) compared to non-treated  
443 cells ( $63.26 \pm 2.74$ % and  $31.48 \pm 4.087$ % respectively; Fig. 7A and B). The pre-  
444 treatment of the epithelial cells with a monoclonal anti-TLR 2 blocking-antibody before  
445 the PGN stimulation, allowed JAr spheroids to adhere to the monolayer to similar levels  
446 ( $58.15 \pm 4.14$ % for RL95-2 and  $33.15 \pm 4.44$ % for MCF7) to those observed in the non-  
447 stimulated control ( $61.81 \pm 2.65$ % for RL95-2 and  $35.01 \pm 3.78$ % for MCF7; Fig. 7A and  
448 B). The treatment with the antibodies did not show an effect on the adhesion of JAr  
449 spheroids to the monolayers (anti TLR 6:  $63.35 \pm 2.48$ % for RL95-2 and  $33.08 \pm 4.28$ %  
450 for MCF7; anti TLR 2:  $61.96 \pm 3.25$ % for RL95-2 and  $36.72 \pm 2.45$ % for MCF7)  
451 whereas the stimulation with FSL-1 and PGN decreased significantly the adhesion of the

452 JAr spheroids (FSL-1:  $45.70 \pm 1.91\%$ ,  $p < 0.0001$  for RL95-2 and  $18.20 \pm 3.66\%$ ,  $p <$   
453  $0.0005$  for MCF7; PGN:  $45.34 \pm 3.51\%$ ,  $p < 0.0001$  for RL95-2 and  $24.00 \pm 2.87\%$ ,  $p <$   
454  $0.0001$  for MCF7).

455

456 *The viability of the epithelial cells was unaltered after TLR activation*

457 The stimulation of the RL95-2 and MCF7 cells with both FSL-1 and PGN did not affect  
458 the viability of the cells. Around 90% of the non-stimulated cells remain viable after  
459 harvesting from the wells. The viability of the cells stimulated with FSL-1 or PGN was  
460 also around 90% of the total population (Figure 8).

461

462 **Discussion**

463 Embryo implantation is a complex event initiated by the adhesion of the trophectoderm to  
464 the endometrial epithelial cells. This is followed by the trophoblast invasion into the  
465 uterine decidua. Two major factors are involved in a successful implantation, a good  
466 embryo quality and proper endometrial receptivity. Any disturbance in the intricate  
467 crosstalk of hormones, cytokines, and adhesion factors during this critical period of time  
468 could lead to implantation failure[32,33].

469

470 In our study using an in vivo murine model, we explored the characteristics of the UH on  
471 pre-implantation and implantation day. We were able to create an inflammatory  
472 environment in the UH by stimulating the innate immune system via TLR 2/6 ligand and  
473 observe how it could affect the tissue structure and impair embryo implantation. The  
474 inflammatory insult in the UH strongly affected the architecture of the different layers of

475 the tissue. This effect could be observed as soon as 4 h of the FSL-1 administration, when  
476 the endometrium is remodeling for suitable embryo receptivity. This might indicate that  
477 the epithelial and stromal cells of the uterus are sensitive to innate immune activation via  
478 TLR stimulation as a protective mechanism for the mother. Stromal cells have been  
479 found necessary to mediate immune responses in different body tissues. They offer a  
480 substrate for the migrating leukocytes and orchestrate part of the adaptive immune  
481 responses[34]. During embryo implantation, the uterine stromal cells provide a scaffold  
482 for migrating cytotrophoblasts and establishment of the spiral arteries. The stromal  
483 sensitivity to respond to inflammatory stimuli has been studied, where administration of  
484 i.p. Poly I:C (TLR 3 ligand) to mice affects stromal decidualization and vascularization of  
485 the uterus[35]. In our experiments the innate immune activation was performed via FSL-1  
486 which is a synthetic diacyl lipopeptide specific ligand for TLR 2/6[36]. It is possible that  
487 the effect on the tissue remodeling will occur regardless of the type of TLR activated. In  
488 fact, the capacity of the endometrial cells to react to foreign entities has been assessed  
489 previously where the exposure of the UH to the toxic compound di-(2-ethylhexyl)-  
490 phtalate (DEHP) is able to affect tissue decidualization and impair embryo  
491 implantation[37]. The current methods to evaluate the estrogenic effects of toxic  
492 compounds on the UH is based on the uterotrophic assay developed in the 1930s. It  
493 estimate the weight of the horns after treatment and gives a description of the  
494 morphological characteristics of the uterine layers and cells[38]. In humans, the criteria to  
495 date the endometrium based on pathologist observations were established by Noyes in the  
496 1950s. It estimates scores for the different morphological changes of the endometrial  
497 characteristics in gland, epithelia and stromal development due to the phase of the

498 cycle[39]. In our morphometry assessment, we were able to measure accurately the areas  
499 of the different UH compartments with the help of the digital imaging tools. Also the  
500 possible differences due to the horn size and sampling along the length of the horn were  
501 normalized with the calculation of the indexes. This technique can potentially be used as  
502 an effective method of estimating toxic effects of chemical compounds on the uterine  
503 horn of different mammalian species, although more standardization in its validity is  
504 required.

505

506         The implantation as mentioned before is an agreement between the maternal tract  
507 and the embryo. Cytokines produced by the maternal tract are fundamental effectors,  
508 which also peculiarly resemble an inflammatory reaction. By profiling the UH secretions,  
509 we were able to observe a disruption in the cytokine levels when the uterine TLR 2/6 was  
510 activated. After 4 h of stimulation (pre-implantation day) three proteins were up-  
511 regulated: MCP-1 (CCL2), IL-1 $\beta$  and IL-1ra. The three of them share important roles in  
512 both embryo implantation and in inflammation. The stimulation of the endometrium  
513 epithelia with IL-1 $\beta$  has been found induce the expression of endometrial adhesion  
514 molecules necessary for embryo apposition and adhesion[40]. MCP-1 has been proven to  
515 be inducible by in vitro stimulation of TLR 2/6 in murine uterine epithelial cells and  
516 TLR4 in uterine epithelial and stromal cells[41]. The increase in IL-1RA production after  
517 24 h of TLR 2/6 stimulation could interfere with the embryo – mother crosstalk  
518 antagonizing IL-1 $\beta$ . The i.p. administration of IL-1RA to mice in the pre-implantation  
519 period reduces drastically embryo implantation[42]. After 24 h of FSL-1 treatment we  
520 observed a distinctive change in pattern of expression of cytokines towards a response

521 type Th17. The Th17 response can lead to autoimmune diseases, chronic inflammatory  
522 disorders and infectious diseases[43]. In the endometrium they have been associated with  
523 cases of unexplained infertility[44,45] and in response to *C. muridarum*, the murine  
524 homologue of *C. trachomatis*[46]. This pathogen can initially be recognized by TLR 2  
525 prior to an adaptive response[16].

526

527         The reduction of embryo implantation confirmed a direct effect of the activation  
528 of uterine TLR 2/6 on embryo implantation. This activation was able to affect the  
529 development of the uterine tissue and the secreted protein profile necessary for  
530 implantation. As observed, the lack of a receptive epithelium to adhere would affect the  
531 apposition and adhesion of the blastocyst. It might be possible that the molecular  
532 characteristics of the endometrial cells could be also affected by this stimulation. It is  
533 unlikely that the treatment of the UH with the TLR 6 agonist could harm the embryos and  
534 could be the reason of embryo implantation loss. A previous report has demonstrated that  
535 the first trimester trophoblast lack the expression of TLR 6 and stimulation does not  
536 change blastocyst viability[47]. The FRT has been proved to be sensitive to TLR  
537 stimulation in vivo, for example the infusion of LPS to the murine UH induces a TLR 4  
538 dependent inflammatory pelvic disease [48]. It has also been observed that the  
539 administration of i.p. LPS to mated mice on day 5.5 could increase fetal loss[49]. The  
540 atrophic stroma observed after TLR 2/6 stimulation, would offer a deficient soil for the  
541 embryo to invade and start its development. This is likely to be a contributing factor for  
542 the increase in embryo resorption observed in our investigation. Similar to this study, it  
543 has been observed that administration of i.p. Poly I:C, TLR 3 ligand can decrease the

544 implantation rate and increase the percentage of resorbed embryos by impairing stromal  
545 decidualization and spiral arteries development[35]. Hence, a potential reason for the low  
546 success rate of implantation after embryo transfer is the simple manipulation of the  
547 uterine horn or endometrium during embryo transfer procedures in human or livestock  
548 that could damage the tissue. This damage could induce a sterile inflammatory process by  
549 the release of danger associated molecular patterns (DAMPs) molecules that are also  
550 recognized by the TLRs, initiating an inflammatory process and leading to embryo  
551 implantation failure. A potential way of clarifying these effects on the murine UH and a  
552 future scope of our research is to use a TLR6 KO mouse. In this way we would be able to  
553 test the effects of TLR activation on both tissue structure and embryo implantation.

554

555 To corroborate our observations in the murine model of embryo implantation, we used an  
556 in vitro human trophoblast spheroid adhesion model as mentioned before. The percentage  
557 of spheroids adhered increased proportionally with time, as observed previously[50,51].  
558 The JAr cells have been described as a very invasive choriocarcinoma cell line[50]. The  
559 interaction of MCF7 cells with first trimester placental explants has been observed  
560 before. The trophoblast explants affected MCF7 proliferation and induced apoptosis in  
561 long co-incubation times. [52]. Our experiments were carried out in short co-incubation  
562 times where we were able to observe adhesion of trophoblast to both epithelial cells.  
563 Nevertheless a better interaction was observed with the endometrial cells as trophoblast  
564 cells adhered them in a higher percentage and faster than the MCF7 cells.

565

566 Stimulation of TLR 2 and 2/6 in the epithelial cells decreased the percentage of attached  
567 spheroids to the monolayers. It is unclear how the TLR activation can influence  
568 endometrial receptivity. The fact that we observed a decrease in trophoblast spheroid  
569 adhesion in both epithelial cell lines after TLRs activation might suggest that the TLR  
570 activation effect relies on modification of cellular adhesion capability. Expression of  
571 surface adhesion molecules, cell polarity or even cytoskeletal arrangement necessary for  
572 the cell-cell interactions might be involved. In our study it was clear that the decrease in  
573 trophoblast adhesion to epithelial cells came from the TLR activation on the epithelial  
574 cells. The inhibition of TLR 6 and TLR 2 with specific antibodies restored the ability of  
575 the JAr spheroids to bind to both FSL-1 and PGN stimulated RL95-2 cells. It is important  
576 to consider that TLR 2 is a particular receptor of the TLR family. It can form homodimers  
577 and heterodimers with either TLR 1 or TLR 6 increasing the range of ligands that TLR 2  
578 can recognize, from bacterial PGN to diacyl and triacyl lipopeptides[53,54]. TLR 2/6  
579 functionality is dependent on the dimerization and the recognition of the ligand by both  
580 TLRs. If one of them is absent, then the activation by the diacylated lipopeptides will not  
581 occur[19,55]. A previous report from our laboratory explored how the stimulation of TLR  
582 5 with Flagellin can induce a similar effect on the adhesion of trophoblast cells to  
583 endometrial epithelial cells[24]. Finally, as shown in our results (Figure 8), the decrease  
584 in spheroid adhesion to the endometrial cells cannot be attributed to the effect of the TLR  
585 stimulation on the viability of the cells. Together these results suggest that the activation  
586 of TLRs will have a detrimental effect on implantation and this might happen regardless  
587 of which TLR molecule is being stimulated.  
588

589 Clinically, endometrial TLR 2 might be playing a very important role in the  
590 recognition of pathogens that cause Sexually Transmitted Diseases (STDs) and infertility.  
591 The most common bacterial transmitted STD caused by the intracellular obligated gram-  
592 negative pathogen *C. trachomatis*. Chlamydia can cause a cervical infection that if  
593 ascends into the upper tract can generate Pelvic Inflammatory Disease (PID) and  
594 infertility[56]. Chlamydia can be recognized by TLR 2 in the intracellular inclusions and  
595 induce the production of IL-8[16]. Furthermore this recognition and the initiation of the  
596 immune responses is dependent on TLR 2 and not TLR 4 and mice lacking TLR 2  
597 showed a reduced oviductal inflammation[57].

598

599 Determining the suitable endometrial conditions for the implantation of the  
600 embryo is a topic under constant research[2,3,5,6,33]. The correct diagnose of infertility  
601 can increase significantly the success of pregnancy for women with fertility problems and  
602 couples undergoing IVF treatment. With further research on the mechanisms responsible  
603 for the effects observed here, it would become possible to design and direct a therapy  
604 targeting the endometrial innate immune system to increase the implantation success. Our  
605 data support the importance of TLRs during the time of implantation suggesting that they  
606 could be used as a potential target in management of infertility cases.

607

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609

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614

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## List of figures

**Figure 1.** Donor and foster mice preparation for embryo transfer and FSL-1 administration scheme. Scheme of super-ovulation of embryo donor mice, foster mother preparation, administration of FSL-1 and blastocyst transfer.

**Figure 2.** Uterine horn morphology and morphometry after TLR 2/6 stimulation. Cross-section of the H&E stained murine UH treated with saline solution or FSL-1 (0.1  $\mu\text{g}/\mu\text{l}$ ) after 4h on pre-implantation day (A) and after 24h on implantation day (C). Images were taken at 40x and 400x magnifications. Morphometric analysis of the different layers of saline and FSL-1 treated UHs: endometrium (Ei), myometrium (Mi), lumen (Li) and glands (Gi) on pre-implantation (B) implantation (D) day. The bars are representative of the mean  $\pm$  SEM from 10 cross-sections of each UH. The statistical comparisons between saline control and FSL-1 treatments were performed with a Mann Whitney t-test where \*  $p \leq 0.05$ . Lu: Lumen, En: Endometrium and My: Myometrium.

**Figure 3.** Production of soluble proteins in the uterine horn lumen stimulated with TLR 2/6 ligand. Semi quantitative profiling of the luminal lavages after 4 (A) and 24 h (B) of saline or FSL-1 treatment (0.1  $\mu\text{g}/\mu\text{l}$ ). Quantification of the production of IL-1 $\beta$ , MCP-1 and IL-1RA after 4 (C) or 24 h (D) of saline (white bars) or FSL-1 (black bars) treatment. The bars on the array represent the mean  $\pm$  SEM of two technical replicates of the pooled flushing samples of saline (n=10) or FSL-1 (n=16) treated UHs. The bar charts of IL-1 $\beta$ ,

MCP-1 and IL-1RA represent the mean  $\pm$  SEM of the logarithmic concentration of the different samples of the UH flushed media. Statistical differences between treatments were analyzed by Mann Whitney t-test (\* $p \leq 0.05$ ).

**Figure 4.** Murine embryo implantation on FSL-1 treated uterine horns. The percentage of total embryos implanted was assessed on day 16 on the control (n=15) and FSL-1 (0.1 $\mu$ g/ $\mu$ l; n=16) treated foster mothers (A). Representative image of implanted and resorbed embryos in the murine UH (B). The percentage of embryos implanted (C) and embryos resorbed (D) was estimated and compared between control and FSL-1 treated UHs. The bars are the mean  $\pm$  SEM of the control and FSL-1 treated foster mice. The statistical comparisons between treatments were performed using a Mann Whitney t-test (\* $p \leq 0.05$ ).

**Figure 5.** Kinetics of adhesion of trophoblast spheroids on epithelial cells stimulated with FSL-1 and PGN. The percentage of adhesion of JAr spheroids to A) endometrial or B) MCF7 cells was determined after a kinetic of adhesion (0.5, 1, 2 and 4h). The adhesion of the spheroids to endometrial untreated cells (●) was compared to the endometrial cells treated (■) with FSL-1 100 ng/ml treated or PGN 10  $\mu$ g/ml treated. Each point represents the mean  $\pm$  SEM of four independent experiments treatments were compared by a two-way ANOVA to test interaction between effects, treatment and co-incubation time. Furthermore the difference between control and treated groups per individual time points was assessed with a Bonferroni multiple comparisons post-test (\* $p \leq 0.05$  was considered significant).

**Figure 6.** Effect of the concentration of FSL-1 and PGN on the adhesion of the JAr spheroids to the epithelial monolayers. The adhesion of JAr spheroids to the A) endometrial or B) MCF7 cells was determined after their pre-treatment with different concentrations of FSL-1 (50, 100 and 200 ng/ml) or PGN (5, 10 and 20 µg/ml). The data represents the mean of 3 independent experiments ± SEM. A multifactorial ANOVA with Bonferroni multiple comparison test was used to compare the stimulated vs. non-stimulated groups (\* $p \leq 0.05$ ).

**Figure 7.** Effects of the inhibition of epithelial TLR 6 and TLR 2 activation on the adhesion capacity of the JAr spheroids. The A) RL95-2 or B) MCF7 cells were pre-treated or not with an IgG anti-TLR6 antibody (10 ng/ml) or IgA monoclonal anti TLR-2 antibody (2 µg/ml) and stimulated with FSL-1 (10 ng/ml) or PGN (10 µg/ml) respectively. The percentage of adhesion to the non-treated or different treated groups was assessed. The data represents the mean of 3 independent experiments ± SEM. A multifactorial ANOVA with Bonferroni multiple comparison test was used to compare the different groups (\* $p < 0.05$ ).

**Figure 8.** Effect of the concentration of FSL-1 and PGN on the viability of the epithelial cells. The viability of A) RL95-2 or B) MCF7 cells was determined after their treatment with different concentrations of FSL-1 (50, 100 and 200 ng/ml) or PGN (5, 10 and 20 µg/ml). The data represents the mean of 3 independent experiments ± SEM. A

multifactorial ANOVA with Bonferroni multiple comparison test was used to compare the stimulated vs. non-stimulated groups (\* $p \leq 0.05$ ).